



MicroRNA-449b Reduces Methylation and Apoptosis-related Gene Expression in Fibroblast Cells Containing Human Insulin Gene

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ABSTRACT

Background: MicroRNA-449b (miR-449b) is a ~22-base pair long nucleotide, that post-transcriptionally affects processes like proliferation, differentiation and apoptosis by directly targeting the *HDAC* and *DNMTs* family genes that regulate DNA methylation.

Methods: This study aimed to investigate the effect of miR-449b mimic, inhibitor and scrambled sequence on buffalo fibroblast cells containing the human insulin (*hINS*) gene. A transgenic buffalo cell line containing human insulin gene (*hINS*) was treated with 40 nM miR-449b mimic and inhibitor by transfection method (lipofectamine-3000).

Result: miR-449b mimic treated cells showed a significant decrease ($P < 0.05$) in the expression of epigenetic-related genes and found a significant reduction ($P < 0.05$) in the expression of *HDAC1*, *DNMT3A* and *DNMT3B* genes. In contrast, *DNMT1* showed no significant change ($P < 0.05$) in their expression level as compared to control whereas miR-449b inhibitor-treated cells found significantly increased ($P < 0.05$) expression levels of *HDAC1*, *DNMT 3A*, *DNMT 3B* and *DNMT1* genes as compared to control. In apoptosis-related genes, miR-449b mimic significantly increased ($P < 0.05$) the expression of *BCL-XL* and *MCL-1*, while miR-449b inhibitor significantly increased ($P < 0.05$) the expression of *MCL-1* gene whereas there was no significant change ($P < 0.05$) in the expression of *BCL-XL*. These findings indicate that treatment of miR-449b mimic on transgenic cells reduces the DNA methylation and apoptosis levels. This reduced methylation level helps in the nuclear reprogramming of transgenic cells. Thus, these cells can be a better choice as donor somatic cells for efficient handmade transgenic cloned embryo production.

Key words: Buffalo embryos, Insulin gene, Micorna-449b, Transgenic cell.

INTRODUCTION

Epigenetic mechanisms are heritable changes in gene expression without any change in the DNA sequence while change in the phenotype (Morales *et al.*, 2017). Epigenetic regulation plays a vital role in cell growth, differentiation, proliferation and embryogenesis. The most significant epigenetic mechanisms include DNA methylation and histone modifications and these processes are mediated by the recently discovered class, the non-coding RNAs and miRNA is one of them (Li *et al.*, 2019). Micro RNAs have been classified as epigenetic modulators as they affect the protein levels of the target mRNAs (Wei *et al.*, 2017). Emerging evidence shows that more than one hundred miRNAs are regulated by epigenetic mechanisms and about one-half of them are modulated by DNA methylation (O'Brien *et al.*, 2018). MicroRNAs are a set of small, non-coding RNAs that have 19-24 nucleotides and are highly evolutionarily conserved. They bind to the 3'-untranslated regions of target mRNAs to either degrade the target or prevent further expression (Cai *et al.*, 2020). In recent years, evidence suggests mature spermatozoon contain coding RNA and non-coding RNAs including miRNA (microRNA), piRNA (Piwi-interacting RNA), endo-siRNA (endogenous small interfering RNA) and tRNA (transfer RNA) and studies have shown that the sperm-borne RNA delivered plays an important role in embryonic development following fertilization (Paulson *et al.*, 2022). MiR-449b is a member

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of miR-449 cluster composed of miR-449a, miR-449b, miR-449c and miR-449d and gene ontology annotations indicate that miR-449 related pathways include DNA damage response, cell cycle and senescence and autophagy in cancer and a loss of function of miR-449 gene in human found gastric tumors as compared to normal tissues (Bou Kheir *et al.*, 2011). It was reported that after over-expression of miR-449b; the viability, proliferation and tumor sphere formation of glioblastoma cells was significantly inhibited (Hou *et al.*, 2020) and earlier studies have established that *HDAC1* is a direct target gene of miR-449b (Guo *et al.*, 2021) and it also affects other genes, including *CDK6*, *c-MYC*, *HDAC1* and *BCL-2*. Overexpression of miR-449b improves the epigenetic reprogramming and reduces

apoptosis in bovine preimplantation cloned embryos. (Wang *et al.*, 2017). However, to the best of our knowledge, the published reports are scanty on the effects of miR-449b treatment (mimics and inhibitors) on transgenic cells (containing the human insulin gene). Therefore, the present study aims to investigate the impact of miR-449b mimic and inhibitor on the expression level of epigenetic and apoptosis-related genes when the transgenic cells containing human insulin gene (*hINS*) are treated with miR-449b mimic and inhibitor.

MATERIALS AND METHODS

All the media and chemicals utilized in this study were procured from Sigma Chemical Co. (USA) and the plasticware was obtained from Nunc (Denmark), unless otherwise mentioned. Fetal bovine serum (FBS) was acquired from Gibco Life Technologies (USA).

Establishment of buffalo fetal fibroblast (BuFF) cell line

Female buffalo fetus obtained from slaughterhouse-based animal was washed twice with normal saline fortified with antibiotics (gentamicin sulfate and penicillin-streptomycin). Then surface of the fetus was washed with 70% ethanol followed by several washings with antibiotic-fortified normal saline. Ear pinna tissue was washed 4-6 times with DPBS containing 50 µg/ml gentamicin sulfate. The biopsies were then cut with the help of a surgical blade into small pieces (~1 mm³) which were then again washed 3-4 times with DPBS followed by the cell culture medium (DMEM supplemented with 2.0 mM L-glutamine, 1% non-essential amino acids, 20% FBS and 50 µg/ml gentamicin sulfate). The explants were cultured into tissue culture flasks in a CO₂ incubator (5% CO₂ in air) at 37°C. The media was then replaced with a fresh medium every 3rd day until the fibroblast monolayer attained 30-40% confluence. Then cell monolayer was washed with DPBS and partially detached by trypsin-EDTA (0.25%) for 2 min to ensure fractional detachment of the fibroblasts while other cells, especially epithelial cells, remained attached to the culture flask. The sub-cultured cells were seeded in a new culture flask and anchored cells were allowed to grow till confluency.

Transfection of buFF cells with pAcISUBC vector (containing human insulin gene)

An expression vector "pAcISUBC" was earlier constructed, had human insulin (*hINS*) gene with beta-lactoglobulin (buBLG) promotor and buBLG 3' UTR into pAcGFP-N1 (Clontech Laboratories Inc, USA) vector backbone (Kaushik *et al.*, 2014). Buffalo fetal fibroblast cells were transfected by nucleofection (AMAXA Biosystem, Germany) with pAcISUBC plasmid containing *hINS* gene. Transfected cells were cultured for the first 24 h in the culture medium containing 800 µg/ml G418 in 4 well culture plates with a medium change every 48 h for 2-3 weeks to obtain transfected cell colonies. After 2-3 weeks of enrichment of transfected cells, transgene integration was confirmed by

PCR amplification of *hINS* gene fragment as well as GFP expression observed under the fluorescence microscope (Mehta *et al.*, 2019).

miR-449b treatment to BuFF cells containing pAcISUBC vector

Transgenic buFF cells (10⁵/well) were seeded in a 6-well plate 24 h before transfection. Transfection of miRNAs was done with lipofectamine-3000 (Invitrogen, USA), according to the manufacturer's protocol. Briefly, miR-449b mimics, inhibitor and scramble sequence (Ambion, USA), individually diluted to 40 nM in serum-free Opti-MEM and lipofectamine 3000 (10 µl/250 µl Opti-MEM) were incubated at room temperature for 15 min. After incubation, the two solutions were mixed thoroughly, incubated for 20 min and added to the cultured transgenic BuFF cells and kept in CO₂ incubator (at 37°C, >95% RH and 5% CO₂ in air). Thereafter, fresh complete medium was added after 4 h of incubation (Tripathi *et al.*, 2024).

Expression of epigenetic and apoptosis-related genes

The miR-449b treated transgenic cells were harvested 48 h later from all three groups (miR-449b mimic-treated, miR-449b inhibitor-treated and untreated control) and the effect of miR-449b was studied on some epigenetic-related (*DNMT1*, *DNMT3A*, *DNMT3B*, *HDAC1*) and apoptosis-related (*MCL-1*, *BCL-XL*) genes by qPCR. For this, total RNA isolation was performed using the Single Cell RNA Purification Kit (NORGEN, Canada) following the manufacturer's protocol and cDNA was synthesized using SuperScript III Kit (Invitrogen, USA). Briefly, the reaction mixture consisted of 100 ng RNA, 1 µl oligo dT, 1 µl 10 mM dNTP mix, 1 µl random primers and 10 µl DNase-/RNase-free water. The mixture was incubated at 65°C for 5 min, followed by a cooling step on ice for 3 min. A master mix containing 4.5 µL of 5X First Strand Buffer, 1 µL of 0.1M DTT and 0.25 µL (50 U) of SuperScript III RT was added. The reaction was performed using the following program: 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. Subsequently, the cDNA was diluted 1:4 (v:v) with nuclease-free water and gene amplification was carried out using Maxima SYBR Green Master Mix (Fermentas, USA) along with primer sets (Table 1). The thermal cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s annealing at 60°C for 30 s and extension at 72°C for 30 s. The expression levels of the target genes were normalized using internal control genes, with *GAPDH* and *B-TUBULIN* (Shyam *et al.*, 2020). Details of primers used in gene expression analysis is present in Table 1.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7 software. One-way analysis of variance was conducted and Student's t-test was employed for comparing the means of different groups.

RESULTS AND DISCUSSION

Transfection of BuFF cells with pAcISUBC vector and their enrichment

The fibroblast cells from cultured ear tissues started to migrate after 3-5 days of culture (Fig 1A). When these cells reached 30-50% confluency, then were sub-cultured by partial trypsinization and transferred to a new culture flask (Fig 1B). Buffalo fetal fibroblast cells were transfected with pAcISUBC vector containing *hINS* gene. After the nucleofection, transfected cells were grown in selection media containing geneticin (800 µg/ml), only transfected cells survived in selection media. After 15-20 days of selection, a pure population of transgenic cells was obtained (Fig 2). Transfected cells showed a normal morphology and GFP expression. RT-PCR of these cells showed a 275 bp human insulin gene fragment

amplification, indicating transgene integration in the genome of cells (Fig 3).

Exogenous miR-449b treatment to transgenic BuFF cells

After geneticin selection, transgenic cells were transfected with miR-449b mimics, inhibitor and scramble sequences. After transfection, on culture, all three groups of cells showed normal cell proliferation, morphology and normal cell senescence. Transfection of miR was confirmed by red fluorescence produced through TAMARA (5-carboxy tetramethylrhodamine) dye tagged with scramble sequences (Fig 4).

Effect of miR-449b on gene expression in transgenic cells

The transgenic cells (containing human insulin gene) after subculture showed a normal growth pattern and about 70-80% of cells showed red fluorescence (control tagged with

Table 1: Details of primers used in gene expression study.

Gene	Sequence	Product size (bp)	Annealing temperature (°C)	Acc. No
<i>hINS</i>	F-CAAGATCTGCCATGGCCCTGTGG R-CTTCTGCCGGGATCCCTCCAGGGC	275	56	NM_000207
<i>HDAC1</i>	F-ATCGGTTAGGTTGCTTCAATCTG R-GTTGTATGGAAGCTCATTAGGGA	168	60	BT030718.1
<i>DNMT1</i>	F-CTCAGAAGGGAGATGTGGAG R-TAGTAGTCACAGTAGCTGAGGA	138	60	NM182651.2
<i>DNMT3A</i>	F-GTGCTGTCTCTATTCTGATGG R-CCATTCCTGGATATGCTTCTG	188	58	NM001206502.1
<i>DNMT3B</i>	F-AGCGGGACATCTCTCGGTTTT R-TCCTATTGAACTCCAGGCAGTC	174	60	NM181813.2
<i>BCL-XL</i>	F-TTGTTGGCCTTTTTCTCCTTC R-GATCCAAGGCTCTAGGTGGT	128	60	NSBTAT00000008572
<i>MCL-1</i>	F-TCGGAAACTGGACATCAAAA R-CCACAAAGGCACCAAAAGAA	128	58	ENSBTAT00000020159
<i>β-ACTIN</i>	F-GAGAAGTCCGAGTTGAGTTTGAA R-GGCTCGTAGTGCAAATGAAGAGT	191	60	NM001034035.2
<i>GAPDH</i>	F-TCAAAGAAGGTGGTGAAGCAG R-CCCAGCATCGAAGGTAGAAG	123	58	NM001034034.2

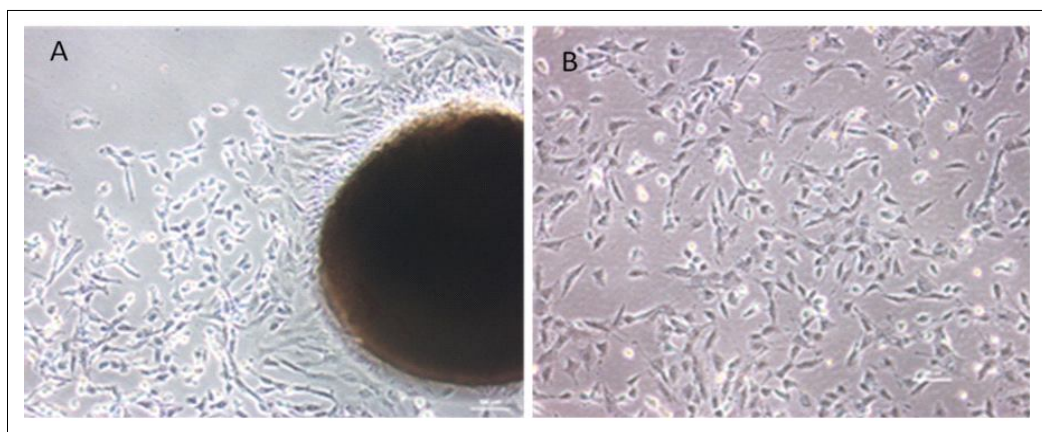


Fig 1: Migration of buffalo fetal fibroblast cells from tissue explant on day 5 (A); fetal fibroblast cells at passage 5 (B).

TAMARA fluorescence dye). Transgenic cells treated with miR-449b mimic, inhibitor and control were harvested after 48 h of treatment and total RNA was isolated and converted into cDNA. The expression level of epigenetic-related genes was found significantly ($P < 0.05$) reduced, *i.e.* *HDAC1* (1.9

fold), *DNMT 3A* (2.5 fold) and *DNMT 3B* (2 fold), while, *DNMT1* showed no significant change ($P < 0.05$) in their expression level as compared to control. Whereas, miR-449b inhibitor-treated cells found significantly increased ($P < 0.05$) expression level of *HDAC1* (5 fold), *DNMT 3A* (3

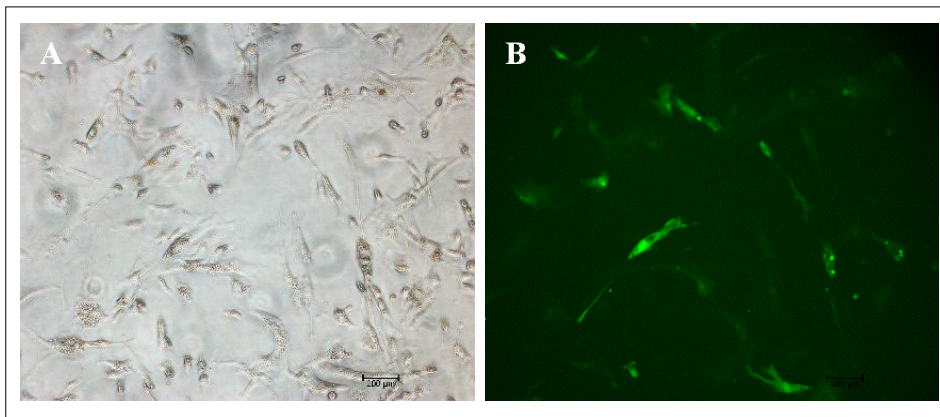


Fig 2: Transfected fibroblast cells after 48 h of nucleofection in bright light (A); and showing GFP expression in fluorescent light (B).

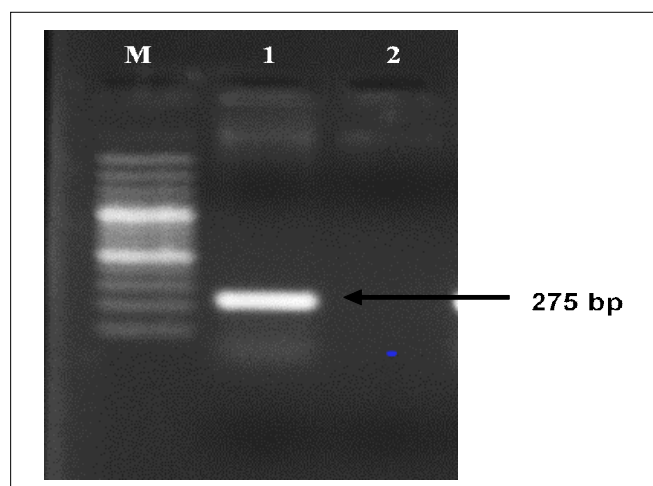


Fig 3: Amplification of human insulin gene (275 bp) in buffalo transgenic cell line (Lane-1); negative control (Lane-2) and 100 bp marker (Lane M).

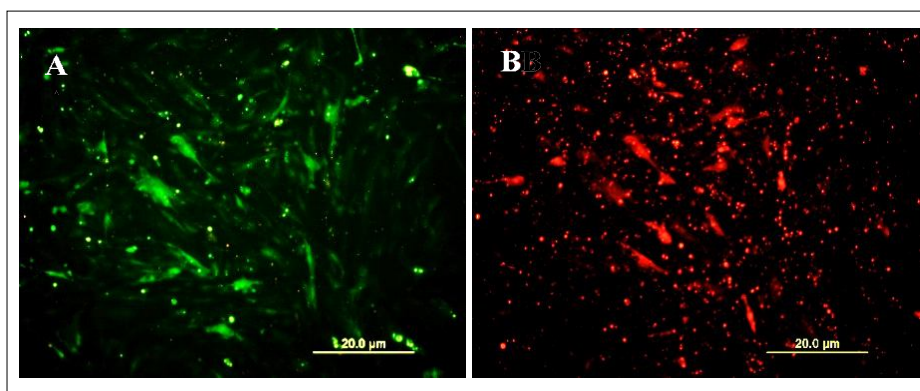


Fig 4: Transgenic cells showing GFP expression (A), expression of TAMARA dye labelled with scrambled sequences (B). The scale bar represents 20 μm.

fold), *DNMT 3B* (7 fold) and *DNMT1* (7 fold) as compared to control (Fig 5). Another apoptosis-related genes, miR-449b mimic significantly increased ($P<0.05$) the expression level of *BCL-XL* (2.5 fold) and *MCL-1* (2.8 fold) while miR-449b inhibitor significantly increased ($P<0.05$) the expression level of *MCL-1* (2.8 fold) while no change ($P<0.05$) in the expression level of *BCL-XL* (Fig 6).

Epigenetic changes coordinate with chromatin remodelling and are heritable patterns of gene expression (Wang *et al.*, 2020). Inherent epigenetic control is mainly maintained by chemical modifications, which are propagated through mitosis and, in some cases, through meiosis including DNA methylation/hydroxyl methylation and post-translational modifications of histone tails (e.g. acetylation, phosphorylation, methylation), as well as chromatin structure and nuclear architecture (Czernik *et al.*, 2019). The field of epigenetics has nowadays been extended to small noncoding RNAs that affect gene

expression, which includes microRNAs (miRNA), small interfering RNAs (siRNA) and piwi-interacting RNAs (piRNAs) (Reza *et al.*, 2019). Sperm miRNAs may influence embryo development and therefore, might be related to in vitro production of embryos. miR-449b was highly expressed in bovine sperm (Du *et al.*, 2014), which is delivered into oocytes to participate in the development of embryos after fertilization. miR-449b is reported to regulate the epigenetic pattern by targeting DNMTs and HDAC (Wang *et al.*, 2017).

After transfection with miR-449b mimics, inhibitor and scramble sequences, all three groups of cells showed normal morphology and growth pattern in all three groups and transfection of miR was confirmed by red fluorescence produced by TAMARA. Wang *et al.* (2017) reported that target gene predictions indicated that *BCL-2*, *CDK6*, *c-MYC*, *HDAC1*, *NANOG* and *CCND1* might be the target genes of miR-449b. Several studies indicated that miR-449b may

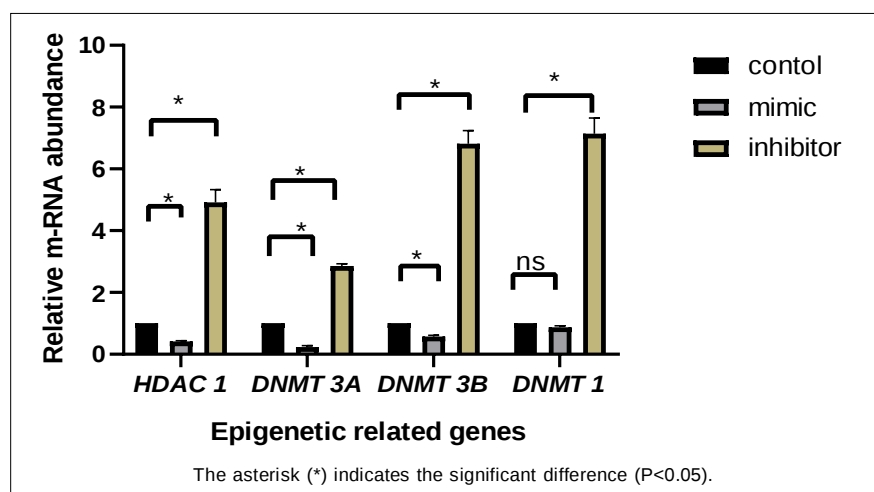


Fig 5: Relative mRNA abundance of some epigenetic-related genes in miR-449b treated transgenic buffalo fetal fibroblast cells.

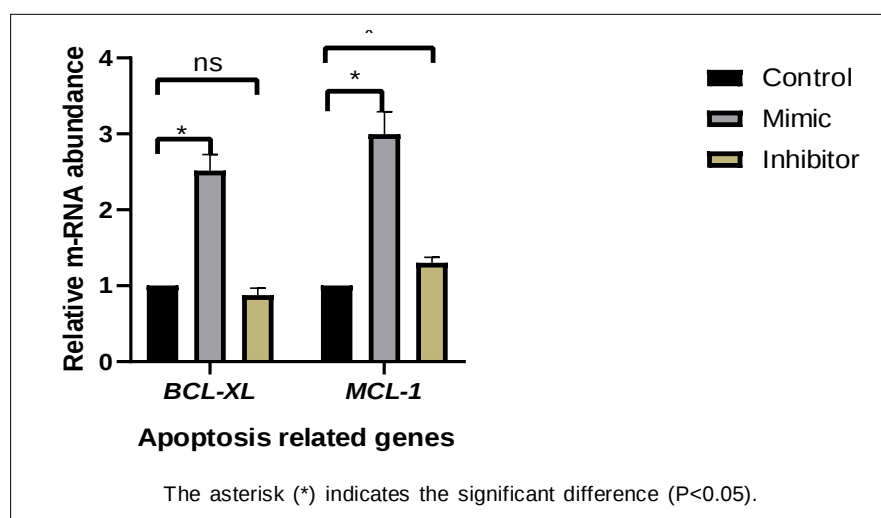


Fig 6: Relative mRNA abundance of some apoptosis-related genes in miR-449b treated transgenic buffalo fetal fibroblast cells.

be involved in histone deacetylation; influenza-induced expression of miR-449b interacted with the histone deacetylase *HDAC1* to alter IFN- β gene expression (Buggele *et al.*, 2013). MiR-449b is also involved in functions and pathways of cell cycle and apoptosis (Wang *et al.*, 2017). The present study showed that the expression levels of *HDAC1*, *DNMT 3A*, *DNMT 3B* and *DNMT 1* were significantly reduced ($P < 0.05$) when transgenic cells were treated with miR-449b mimic and were significantly ($P < 0.05$) increased when treated with miR-449b inhibitor except for *DNMT1*. These results are consistent with the findings of Bou Kheir *et al.* (2011). Wang *et al.* (2017) treated miR-449b mimic with bovine SCNT embryos and found *HDAC1* expression level significantly decreased as compared to control group indicating that miR-449b plays an important role in the development of bovine SCNT embryos. *HDAC1*, could restore the positive charge of histone, which inhibits the gene transcription and regulates the gene expression. *HDAC1* was verified as one of the important target genes of miR-449b and suggesting that miR-449b delivered in the sperm might improve the acetylation level of histone by down-regulating the expression of *HDAC1* promoting the cellular reprogramming (Wang *et al.*, 2017). A similar observation was also made in the present study where miR-449b affected the epigenetic-related genes but *HDAC1* was proved as one of the potent target genes of miR-449b. Apoptosis level increases when cells were transfected with a plasmid vector but once the transfected cells were selected and further propagated, there was not much variation in their growth pattern. Apoptosis was found to be higher in transgenic cells compared to non-transgenic cells (Mehta *et al.*, 2018). A higher level of apoptosis in transgenic cells was correlated with the higher expression of pro-apoptotic and cell cycle checkpoint-related genes in transgenic cells as compared to non-transgenic cells (Mehta *et al.*, 2018) and also found that the lower pregnancy and live birth rate obtained with transgenic embryos could be due to higher level of apoptosis in their cells (Gouveia *et al.*, 2020). Wang *et al.* (2017) reported that the expression level of *BCL-XL* returned to the level of IVF when bovine fetal fibroblast was over expressed with miR-449b through a doxycycline (dox) induced expression system was used as a nuclear donor for SCNT confirming that miR-449b help in regulation of cellular apoptosis. The present investigation, it was found that the expression level of anti-apoptotic genes (*BCL-XL* and *MCL-1*) were significantly increased ($P < 0.05$) as compared to control when treated with miR-449b mimic, which was similar to the earlier studies.

CONCLUSION

This study indicates that miR-449b mimic reduces both DNA methylation and apoptosis levels by altering the gene expression profile in buffalo fetal fibroblasts containing human insulin gene and improving their nuclear reprogramming. Thus, the treated cells can be a better choice as donor cells for transgenic animal production.

Conflict of interest

I hereby declare that there is no conflict of interest related to the authorship, research, or publication of this manuscript.

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